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Differential Expression of Intracisternal A-Particle Transcripts in Immunogenic versus Tumorigenic S49 Murine Lymphoma Cells

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Tumorigenic S49 mouse lymphoma cells (T-25) were compared to their nontumorigenic (immunogenic) substrate-adherent descendants (T-25-Adh), using the differential display technique. A 784-bp fragment with 92% sequence homology to the intracisternal A-particle (IAP) element family was isolated from the latter cells. IAP sequences are endogenous, noninfectious retroviral elements that can undergo transpositions and act as mutagens. Expression of IAP transcripts (as detected by the isolated fragment) was 5- to 10-fold higher in T-25-Adh cells than in T-25 cells. IAP RT-PCR cDNA clones derived from the immunogenic T-25-Adh cells, but not from T-25 cells, contain two distinctive motifs: (i) a motif characteristic of IAP elements expressed in lymphoid cells (lymphocyte specific, LS); (ii) a nonapeptide sequence known to stimulate cytotoxic T lymphocytes in a leukemia cell line expressing IAP sequences. In addition, expression of transcripts containing these motifs is enhanced in the immunogenic cells as opposed to the tumorigenic cells. Furthermore, one of the IAP elements (belonging to the LS1 subfamily) is specifically hypomethylated in the DNA of the immunogenic cells. The above-mentioned relationship was strengthened when tumorigenic revertants derived from T-25-Adh cells, as well as independently selected tumorigenic and immunogenic S49 sublines, were studied. In all cases, enhanced immunogenicity was linked to the up-regulation of specific IAP elements. No transpositions of LS1 elements were observed among the different sublines studied. These findings suggest that, in the S49 lymphoma, selectively expressed IAP retroviral elements may function in a tumor suppressive capacity by affecting the immunogenic potential of these cells.

INTRODUCTION

Intracisternal A-particles (IAP) are defective retroviruses encoded by endogenous proviral elements. There are approximately 1000 IAP-related elements per haploid mouse genome, but only a limited number are transcribed in normal cells (Mietz *et al.,* 1992). These elements are capable of transposition, as a consequence of which they may either activate or inactivate genes located adjacent to their integration site. Indeed, transpositions of IAP are known to be mutagenic when activation of oncogenes (Kuff, 1990; Lee *et al.,* 1999), growth factors (Lee *et al.,* 1999), or cytokines (Lee *et al.,* 1999; Wang *et al.,* 1997) or inactivation of tumor suppressor genes (Xiao *et al.,* 1995) occurs. Full-length elements are 7.2 kb long, but there are also internally deleted elements that are assigned into subgroups. In most cases transpositions involved deleted type IΔ1 IAP elements, which encode 5.4-kb transcripts (Kuff and Lueders, 1988).

High-level expression of IAP has been described in murine transformed cell lines as well as during normal mouse embryogenesis. Extensive hypomethylation of IAP is found in a variety of tumors. Selective activation of IAP is thought to stem from the methylation status of the LTR (Falzon and Kuff, 1991; Morgan and Huang, 1984), although the preferential presence of or the response to transcription factors also plays a role (Falzon and Kuff, 1990). Very few IAP elements are hypomethylated in normal cells and these have been shown to belong to distinctive IAP element subfamilies that are expressed in normal B and T lymphocytes (Mietz *et al.,* 1992). These transcripts contain a 9-nucleotide motif, named LS (for lymphocyte specific), in the R region that is used to classify IAP LTRs from normal lymphocytes into three classes, LS1, LS2, and LS3.

In our laboratory, we have selected several variants of the S49 murine T cell lymphoma, manifesting different tumorigenic and immunogenic characteristics. Highly tumorigenic (median survival 14 days) suspension-borne variants (named T-25) were selected from parental S49 cells, following 25 consecutive *in vivo* passages (Hochman *et al.,* 1981, 1984). From T-25 cells, substrate-adhesive variants, named T-25-Adh, were selected. T-25-Adh cells showed impaired tumorigenicity and enhanced immunogenicity (protection against a challenge with tumorigenic T-25 cells) in immunocompetent mice (Hochman *et al.,* 1981, 1984, 1990; Mador *et al.,* 1997). We have applied several approaches to study the differences between T-25 and T-25-Adh cells. Thus, we described a

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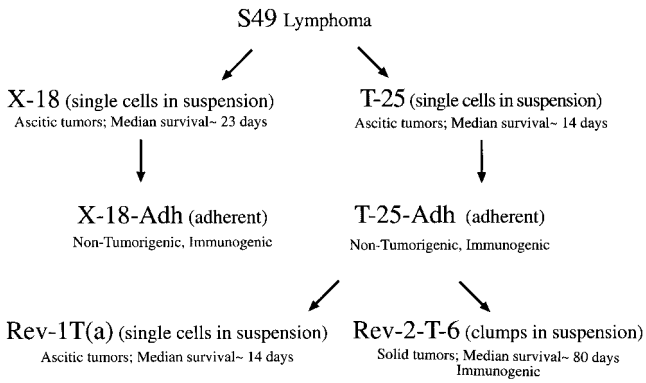

FIG. 1. Relationship between different sublines of the S49 lineage. *In vitro* growth characteristics are given in parentheses.

distinct mutation, specific to each cell line, in the p53 tumor suppressor gene (Bergel *et al.,* 1993). It was also observed that mouse interferon-α (IFN-α) and IFN-β selectively affect the morphogenesis of intracellular MMTV-related precursors in T-25-Adh cells, thereby enhancing their immunogenicity (Mador *et al.,* 1997). Additionally, a novel protein (p21) derived from the *env* precursor of the mouse mammary tumor virus is expressed in nucleoli of T-25 cells but not in T-25-Adh cells (Hoch-Marchaim *et al.,* 1998).

In the present study, we applied the differential display technique (Welsh *et al.,* 1992) to further investigate differences of potential biological significance between T-25 and T-25-Adh cells. These cells are particularly suitable for this approach since the latter were derived from the former via a spontaneous selection process and not through induced mutagenesis, while they are phenotypically distinct in terms of cell adhesion, tumorigenicity, and immunogenicity (Hochman *et al.,* 1984). We demonstrate enhanced expression of the LS family of IAP in T-25-Adh cells compared to T-25 cells. In fact, increased IAP expression is inversely correlated to tumorigenicity and directly correlated to immunogenicity also in other independently selected S49 sublines isolated in our laboratory. We also show that a specific LS1-type IAP element is hypomethylated in the sublines that express high levels of IAP.

RESULTS

The relationship among the different S49 sublines used in this study is depicted in Fig. 1 (see Materials and Methods for derivation of cells). S49 lymphoma cells have been selected *in vivo* for highly tumorigenic, suspension-borne cells through multiple passages in mice. These cells have, in turn, been selected for an immunogenic, nontumorigenic adhesive cell line simply through spontaneous *in vitro* selection for cell-substrate adhesiveness over several generations (Fig. 1). Both of these selection processes have been repeated with similar resulting cell phenotypes, indicating the reproducibility of this method (Fig. 1). Moreover, UV irradiation of T-25-Adh cells followed by *in vitro* selection for growth in suspension culture allowed the selection of revertant, tumorigenic cell lines. We were able to isolate both a highly tumorigenic (single cell) suspension-growing cell line and a less tumorigenic cell line, which grows in suspension as clumps (Fig. 1). It is noteworthy that the latter subline, named Rev-2-T-6, is also immunogenic, since mice surviving inoculation of these cells are protected against a subsequent challenge with tumorigenic T-25 cells (Braun and Hochman, unpublished data). We, thus, have at our disposal a wide spectrum of cell lines, constituting a full cycle from tumorigenic to nontumorigenic cells and back to the tumorigenic state, all derived from the parental S49 cell line.

Differentially expressed mRNA in T-25-Adh cells

Differential display was performed to analyze differentially expressed genes in the nontumorigenic, substrate adherent T-25-Adh cells in comparison with the tumorigenic, suspension-borne T-25 cells (Fig. 2). A high degree of similarity exists between the two cell lines, as shown by the preponderance of identical

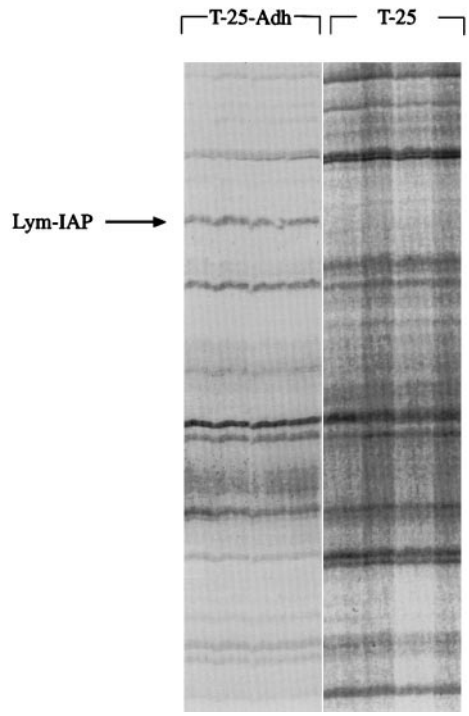

FIG. 2. Differential display of T-25-Adh and T-25 cells. mRNA was isolated, reverse-transcribed, and PCR-amplified in two independent reactions shown in duplicate. The 17-mer primer 5′-CCTCCGCGAGATCATCT-3′ was used in the presence of ^{35}S-dATP. The PCR products were separated on a 4% acrylamide denaturing gel. The arrow indicates a fragment (Lym-IAP) present in the nontumorigenic T-25-Adh cells that was further analyzed.

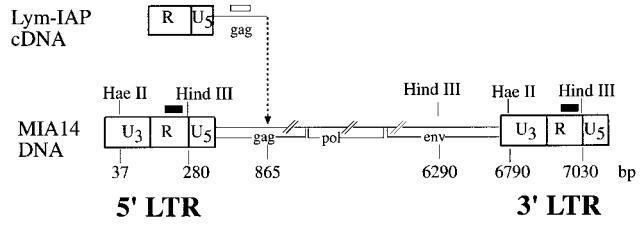

FIG. 3. Location of Lym-IAP cDNA along the MIA14 IAP genomic element. Alignment of Lym-IAP with MIA14 IAP [(Mietz *et al.,* 1987), GenBank Accession No. M17551] starts at position 222 of MIA14 IAP. *Hae*II and *Hind*III sites are indicated (see also Fig. 7). LS sequences are indicated as solid boxes (see also Fig. 4). The sequence of a putative nonapeptide (absent from MIA14) is indicated as an open box (see also Fig. 8).

bands in this and other experiments (not shown) using different arbitrary primers. However, several differentially expressed bands are evident in each cell line. In this work, one 784-bp band, named Lym-IAP (Fig. 2), was eluted from the gel, amplified, cloned into the pCRII vector, and verified by Northern blot analysis to be differentially expressed in T-25-Adh cells (see below).

Sequence analysis of cDNAs

Sequencing of the Lym-IAP cDNA followed by databank search revealed strong similarity with the DNA of endogenous defective retroviruses that belong to the murine IAP element family (see Fig. 3). Homology of 92% over 714 bp was found with the prototype MIA14-IAP sequence (Fig. 4) (Mietz *et al.,* 1987). The Lym-IAP

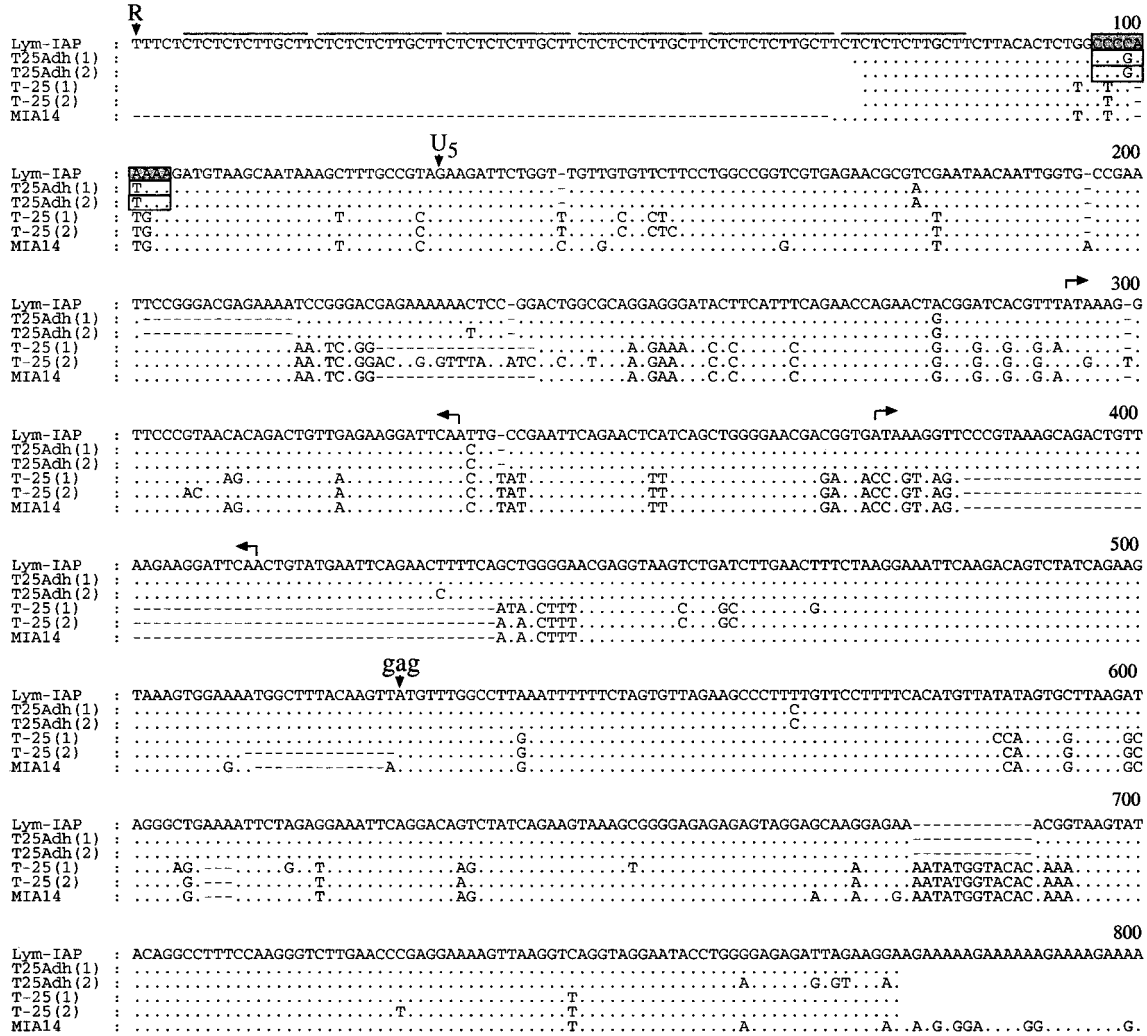

FIG. 4. Nucleotide sequence comparison of IAP cDNA clones derived from S49 cells and the MIA14 IAP element. Sequences compared were as follows: Lym-IAP (GenBank Accession No. AF303450); RT-PCR clones T-25-Adh(1) and T-25-Adh(2) derived from the immunogenic T-25-Adh cells (GenBank Accession No. AF303451 and AF303452, respectively); RT-PCR clones T-25(1) and T-25(2) derived from the tumorigenic T-25 cells (GenBank Accession No. AF303453 and AF303454, respectively); the genomic element MIA14 [(Mietz *et al.,* 1987), GenBank Accession No. M17551]. CT repeat sequences in the R region are indicated by an overline. Shaded and open boxes indicate LS1 and LS2 regions, respectively. R, start of the R region; U_5, start of the U_5 region; gag, start of the gag gene. Arrows indicate the start (↱) and end (↰) of a sequence present in one copy in the T-25 clones, but duplicated in the T-25-Adh clones. Dots indicate identical bases; dashes represent gaps introduced to enhance alignment.

sequence spans the MIA14-IAP sequence from the R region in the 5′ LTR through the first 255 nucleotides of the gag gene (Fig. 4).

The R region of IAP demonstrates extensive variability due to duplication of a CT-rich region (Christy *et al.,* 1985). The R region of Lym-IAP contains six copies of the CT-rich 13-nucleotide repeat, CTCTCTCTTGCTT (Fig. 4). This sequence was previously shown to exist in two to six copies arranged in tandem in IAP cDNA clones derived from normal LPS-stimulated lymphocytes (Mietz *et al.,* 1992) and from BL6 melanoma cells (Li *et al.,* 1996). These repeats are followed by a 9-nucleotide motif (named LS) in the R region. These LS motifs appear as three subtypes, LS1 (CCCCAAAAA), LS2 (CCCGATAAA), and LS3 (CCCCATAAA), which are used to classify IAP from normal lymphocytes into the three corresponding classes: LS1, LS2, and LS3. Oligonucleotide probes that distinguish these three classes detect a limited number of IAP proviral copies in mouse genomic DNA (Mietz *et al.,* 1992).

The Lym-IAP sequence was further compared (Fig. 4) to cDNA clones derived from the immunogenic T-25-Adh cells, named T-25-Adh(1) and T-25-Adh(2), as well as cDNA clones derived from the highly tumorigenic T-25 cells, named T-25(1) and T-25(2). These clones were obtained through RT-PCR using primers constructed from the 5′ and 3′ ends of Lym-IAP (see Materials and Methods). The cDNA clones derived from T-25-Adh cells contain LS motifs. Clone Lym-IAP contains the LS1 motif CCCCAAAAA, while clones T-25-Adh(1) and T-25-Adh(2) contain the LS2 motif CCCGATAAA. The clones derived from T-25 cells, as well as MIA14, contain a sequence different from the LS motif in this region (Fig. 4).

Additional differences in sequence are manifested between the cDNAs expressed in T-25-Adh cells and those expressed in T-25 cells, while the clones within each group share a high degree of similarity. These differences are evident in the region that spans from nucleotide 217 to the beginning of the gag gene (Fig. 4). Another difference between the two groups is the sequence at positions 293–333, present in only one copy in the T-25 clones, but duplicated at positions 374–413 in the T-25-Adh clones. This region is known to be variable and to contain direct and inverted repeats (Christy *et al.,* 1985).

Immunogenic sublines express high levels of IAP transcripts

Messenger RNA from T-25 and T-25-Adh cells was analyzed using the Lym-IAP fragment as a probe (Fig. 5). This probe detected two transcripts: a major transcript of 5.4 kb and a minor transcript of 7.2 kb in both T-25 and T-25-Adh cells. The expression of the 5.4-kb transcript was 5- to 10-fold higher in T-25-Adh cells than in T-25 cells, as analyzed by densitometry. Low-level expression of IAP transcripts was also found in an ascites tumor of T-25 cells (not shown), suggesting that the decreased expression is not due to the *in vitro* culture conditions of these cells.

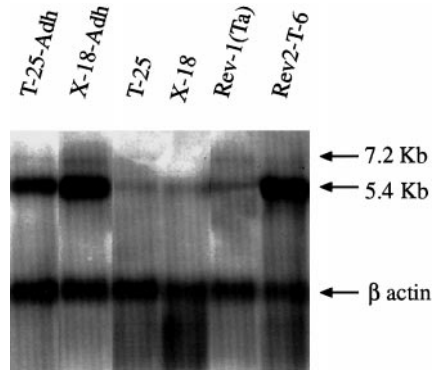

FIG. 5. Northern blot analysis of mRNA from S49 sublines hybridized to the Lym-IAP fragment and β-actin.

To examine IAP expression in our lymphoma model, we analyzed its expression in the other S49-derived sublines (Fig. 1). In addition to T-25-Adh, both X-18-Adh and Rev-2-T-6 express high levels of IAP transcripts (Fig. 5). On the other hand, the tumorigenic S49 sublines X-18 and Rev-1(Ta) (Fig. 1) demonstrate decreased expression of the 5.4-kb transcripts, similar to T-25 cells. It is evident from the results that immunogenic sublines (T-25-Adh and X-18-Adh) express high levels of IAP transcripts, while their parental tumorigenic sublines (T-25 and X-18) do not. Moreover, during the selection for tumorigenic revertants the capability of expressing IAP transcripts was lost. Rev-2-T-6 cells, which express high levels of IAP transcripts, constitute a special case. On one hand, these cells are tumorigenic, albeit less aggressive than T-25, X-18, and Rev-1(Ta). On the other hand, they are capable of immunizing mice against the highly tumorigenic cells.

LS-type IAP elements are highly expressed in the immunogenic sublines

Northern blot analysis using the LS2 sequence as a probe (Fig. 6) detects a 5.4-kb transcript in the immunogenic T-25-Adh, X-18-Adh, and Rev-2-T-6 mRNAs, while only a faint band is seen in the highly tumorigenic T-25, X-18, and Rev-1T(a) mRNAs. When LS1 was used as a probe, both 5.4- and 7.2-kb transcripts were detected. The levels of the 5.4-kb transcripts were higher in T-25-Adh than in T-25 mRNA (not shown). Thus, the selective expression of IAP seen in the immunogenic sublines with the Lym-IAP probe is also seen when the LS sequence was used as a probe. LS1 transcripts of 5.4 and 7.2 kb and the LS2 transcript of 5.4 kb are typically expressed in normal lymphocytes (Mietz *et al.,* 1992).

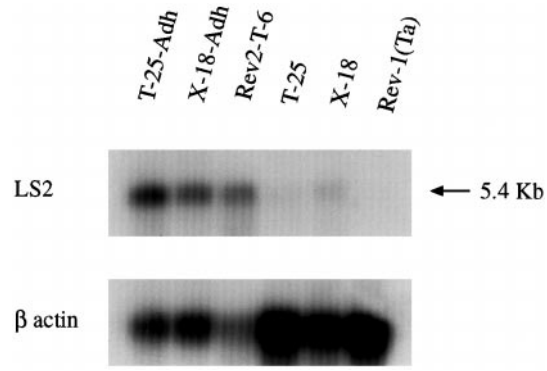

FIG. 6. Northern blot analysis of mRNA from S49 sublines hybridized to LS2 oligonucleotide probe and β-actin.

LS1-type IAP elements are located at the same genomic positions in both the immunogenic and the tumorigenic sublines

Based on the sequence analysis described above, we used the LS1-oligo probe to examine the various S49 cell lines for possible transpositions of IAP in the genome (Fig. 7a). As IAP have a conserved *Hin*dIII site just 3′ to the LS element in the LTR, digestion with this enzyme results in fragments that represent IAP 5′-flanking sequences (see Fig. 3). Thus, each fragment on the gel represents a single LS1 element locus out of 31 that are present in the BALB/c mouse genome (Mietz and Kuff, 1992). The present analysis did not show any differences in the number or position of IAP LS1-type elements in the majority of DNAs. The one exception is Rev-1T(a) cells, whose DNA showed both deletions and additions of LS1 IAP loci.

High-level expression of IAP is correlated with hypomethylation of a specific LS1-type IAP element in the immunogenic sublines

Development of the oligonucleotide probes that detect a restricted number of IAP elements has made it possible to carry out an analysis of the hypomethylation state of individual loci on conventional one-dimensional gels (Lueders *et al.,* 1993a). This method was used to show that established plasmacytomas exhibit a characteristic pattern of IAP hypomethylation when compared to normal liver cells (Lueders *et al.,* 1993a). The technique was also used to show that similar patterns of hypomethylation are seen in established and primary mouse plasmacytomas (Lueders and Kuff, 1995). A single conserved methylation-sensitive *Hae*II site in the LTR (see Fig. 3) makes it possible to assess the methylation status of individual IAP loci. When the restriction fragment pattern of a *Hin*dII digest is compared with that of a *Hin*dIII + *Hae*II digest (Fig. 7b, compare lanes H to lanes H/Ha), any hypomethylated fragments will disappear or become shorter after *Hae*II digestion. As can be seen (Fig. 7b, marked with solid arrowheads), a single fragment is

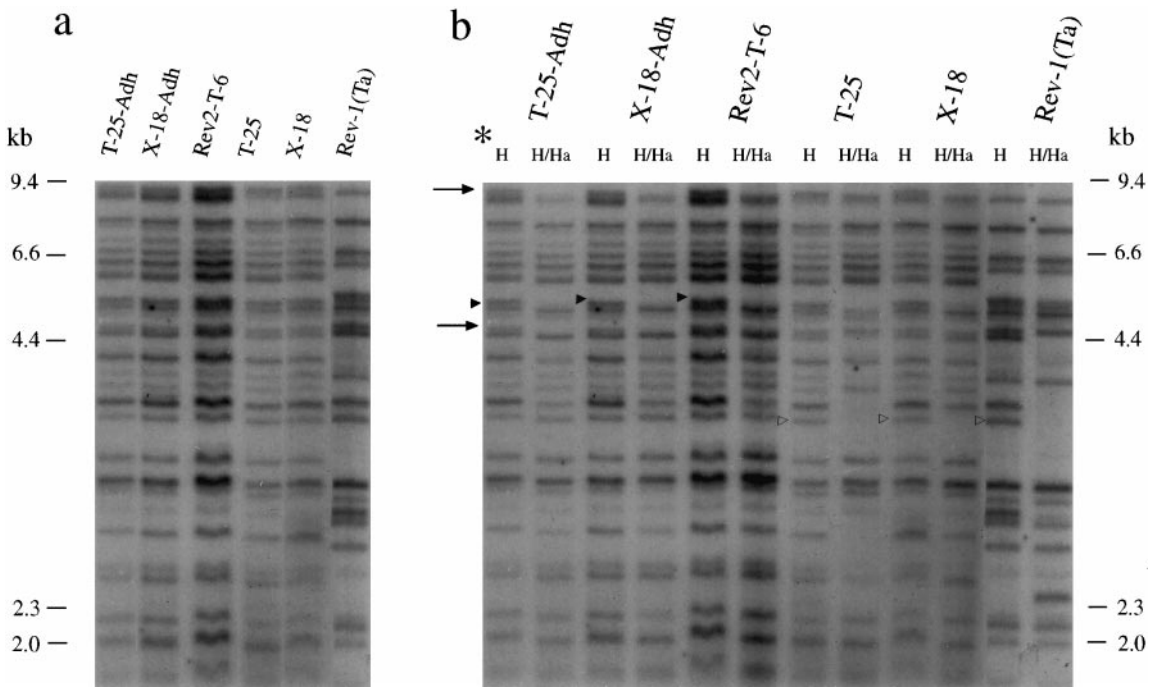

FIG. 7. Genomic location and methylation status of IAP LS1 elements. (a) DNA from S49 sublines was cut with *Hin*dIII and hybridized to LS1 oligonucleotide probe. (b) DNA from S49 sublines was cut with *Hin*dIII alone (H) or with *Hin*dIII and *Hae*II (H/Ha) enzymes and hybridized to the LS1 probe. **Hin*dIII lanes are the same in both a and b. A hypomethylated fragment specific for T-25-Adh, X-18-Adh, and Rev-2-T-6 is marked with solid arrowheads. A hypomethylated fragment (cut with *Hae*II) specific for T-25, X-18, and Rev-1T(a) is marked with open arrowheads. Hypomethylated fragments common to all sublines are marked with arrows.

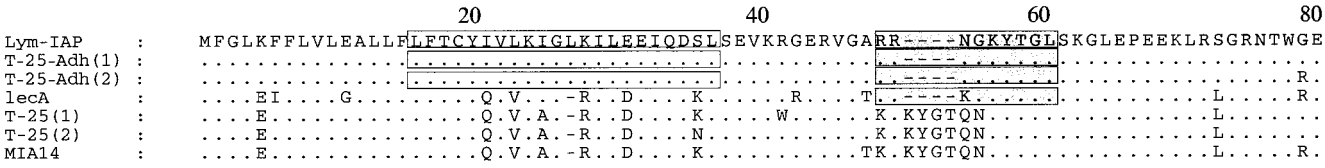

FIG. 8. Predicted amino acid sequence comparison of the gag gene (first 80 amino acids) from IAP cDNA clones derived from S49 cells with MIA14 IAP and LecA IAP elements. Sequences compared were the same as those described in the legend for Fig. 4, with the addition of LecA, a leukemia-derived IAP element [(de Bergeyck *et al.,* 1994), GenBank Accession No. S74315]. Shaded boxes indicate the LecA nonapeptide and its homologues. An open box indicates a leucine-zipper motif.

hypomethylated in the immunogenic T-25-Adh, X-18-Adh, and Rev-2-T-6 cells, while a different fragment is hypomethylated in the highly tumorigenic T-25, X-18, and Rev-1-T(a) cells (Fig. 7b, marked with open arrowhead). Two other fragments are hypomethylated in all S49 sublines (Fig. 7b, marked with arrows).

Differences in predicted amino acid sequence among S49 sublines

The deduced amino acid sequence of T-25 and T-25-Adh cDNA clones was compared (Fig. 8). The sequence includes 80 amino acids from the N-terminus of the IAP gag protein. The comparison starts from the first methionine of the p73 gag protein of the MIA14 IAP element (Fig. 8, bottom line). Two regions show distinct sequence differences among the clones. A leucine-zipper motif, known to be part of a nuclear DNA binding site in molecules such as transcription factors, is present in Lym-IAP as well as T-25-Adh(1) and T-25-Adh(2) cDNA clones, but not in T-25(1) and T-25(2) cDNA clones.

Another unifying feature shared by clones Lym-IAP, T-25-Adh(1), and T-25-Adh(2) is a stretch of 9 amino acids (RRNGKYTGL) at position 49–61. A databank search for this nonapeptide-encoding sequence (27 bases) revealed three IAP elements that share this region (GenBank Accession Nos. U70139.1 and S74315 and EMBL Accession No. X04120). Among them is a very similar nonapeptide (8/9 amino acid identity) present in the IAP gag region of a mouse leukemia (LEC) cell line (de Bergeyck *et al.,* 1994) (see Fig. 8). This nonapeptide induced an immune response toward LEC cells by stimulating cytotoxic T lymphocytes (CTLs) (de Bergeyck *et al.,* 1994). It is noteworthy that the cDNA clones derived from the tumorigenic T-25 cells manifest a somewhat different sequence (KRKYGTQNKYTGL), suggesting that expression of the nonapeptide could be instrumental in the regulation of this phenotype. Northern blot analysis using an oligonucleotide probe encoding the nonapeptide sequence detected a 5.4-kb transcript in the immunogenic T-25-Adh, X-18-Adh, and Rev-2-T-6 lanes, corresponding to the IΔ1-type IAP element (Kuff and Lueders, 1988). Only a faint band of 5.4 kb is seen in the tumorigenic T-25, X-18, and Rev-1-T(a) lanes (Fig. 9), thus supporting the sequence data from T-25 and T-25-Adh cDNA clones. From the sequence data of T-25-Adh clones (Fig. 4), it is evident that both LS1 and LS2 IAP transcripts contain the nonapeptide sequence.

DISCUSSION

In the present study we show that, in S49 lymphoma sublines, there exists a linkage between cell adhesiveness, impaired tumorigenicity, and enhanced immunogenicity. The single-cell suspension growing T-25 and X-18 sublines are highly tumorigenic (Fig. 1), while the substrate adhering variants derived from them (T-25-Adh and X-18-Adh, respectively) are nontumorigenic and immunogenic. Furthermore, suspension (single cells) growing revertants (Rev-1-Ta) derived from T-25-Adh cells are highly tumorigenic, similar to the original T-25 cells. Rev-2-T-6 cells constitute a special case that strengthens the above-mentioned linkage. These cells, derived from T-25-Adh cells, grow in suspension as clumps and, therefore, represent partial revertants (cell–cell adherence) between substrate adhering cells and single cells in suspension. These cells also demonstrate partial reversion of tumorigenicity and immunogenicity, insofar as (i) mice inoculated with Rev-2-T-6 cells demonstrate extended survival (Fig. 1) and (ii) mice that do not develop tumors upon inoculation of these cells are immunized against subsequent challenges with parental T-25 cells.

The differential display technique has been used successfully to delineate differences in gene expression

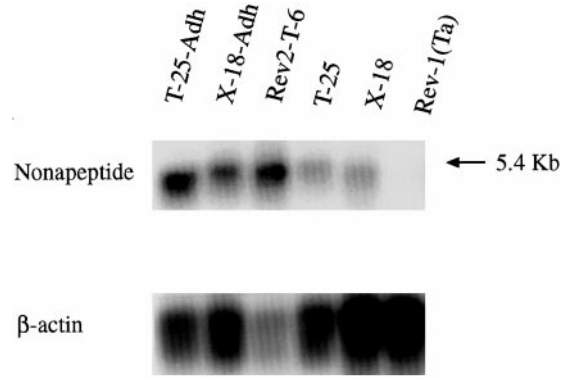

FIG. 9. Northern blot analysis of mRNA from different S49 sublines hybridized to the nonapeptide and β-actin probes.

between closely related cells. In the present study, we have used this technique to investigate the S49 sublines that differ in tumorigenicity, immunogenicity, and adherence. We found that enhanced expression of IAP elements is tightly correlated with impaired tumorigenicity and enhanced immunogenicity. Thus, S49 sublines that are immunogenic (T-25-Adh, X-18-Adh, and Rev-2-T-6 cells) demonstrate a 5- to 10-fold increase in IAP transcripts over S49 sublines that are highly tumorigenic [T-25, X-18, and Rev-1T(a)]. During the transition from tumorigenic to nontumorigenic S49 cells, up-regulation of IAP expression occurs. This is followed by down-regulation of IAP expression when the nontumorigenic S49 cells revert to full-fledged tumorigenicity, thereby constituting a full cycle from tumorigenic to nontumorigenic cells and back to the tumorigenic state. The immunogenic Rev-2-T-6 cells, which are, phenotypically, in an intermediate stage (median survival of ~80 days, grow in suspension culture as clumps and solid tumors *in vivo*), demonstrate an IAP transcript level similar to that of T-25-Adh and X-18-Adh cells. This may indicate that Rev-2-T-6 cells have not undergone the final steps to full-fledged tumorigenicity and suspension-borne single cells, following their selection from mutagenized T-25-Adh cells.

Other studies have also demonstrated an association between IAP expression and *in vitro* or *in vivo* characteristics of different cells. In general, transformed cell lines were found to express larger amounts of IAP transcript than normal tissues, although few studies have attempted to correlate expression levels with varying levels of tumorgenicity within the same system. A correlation similar to ours between reduced tumorigenicity and enhanced expression of IAP was shown in hybrids of myeloma and fibroblast cells, in comparison to their parental myeloma cells. The reduced tumorigenicity of the hybrid cells was postulated to be the result of suppression of the translocated myc gene (Laskov *et al.,* 1991). Increased IAP expression was also seen in *ras*-transformed NIH 3T3 cells treated with azatyrosine (Krzyzosiak *et al.,* 1992). These cells became flat, showed contact inhibition, and scarcely formed tumors in nude mice. It was suggested that IAP, among other activated genes, act cooperatively to counteract ras function. In another study, IAP mRNA levels were 5- to 10-fold higher in SEWA sarcoma cells derived from an ascites tumor than in SEWA cells derived from a solid tumor (Ronai *et al.,* 1992). The cells derived from the ascites tumor grew as multicellular spheroids and as single cells in suspension, while the cells derived from the solid tumors grew attached to the culture plate with a fibroblast-like morphology. These results are in contrast to our findings in S49 cell variants. However, no difference regarding the tumorigenicity of the SEWA sublines was reported. The above-mentioned studies did not provide any information as to which subclass of IAP elements was expressed.

We have shown that the IAP elements selectively activated in T-25-Adh, X-18-Adh, and Rev-2-T-6 cells belong to the LS IAP family. Thus, while T-25-Adh transcripts are derived from the LS1 and LS2 genomic elements, T-25 transcripts are derived from other elements, similar to the prototype MIA14-IAP. The LS1 oligonucleotide reacted with transcripts of 7.2 kb (corresponding to the full-length type-I IAP elements) as well as 5.4 kb (representing IΔ1-type IAP elements), while the LS2 oligonucleotide detected only 5.4-kb transcripts. The same pattern of hybridization with the LS-type IAP transcripts was reported previously in normal BALB/c lymphocytes (Mietz *et al.,* 1992). The study showed that IAP transcripts in normal mouse thymus and activated B cells were largely derived from a few selectively activated and closely related proviral elements. Furthermore, LS elements are activated in both LPS-stimulated spleen cells and several plasmacytomas. In normal lymphocytes, however, the LS subpopulation is the major transcriptionally active group of IAP elements in the thymus of several inbred mouse strains (Mietz *et al.,* 1992). The fact that, in our study, the pattern of LS1 and LS2 expression was similar to that found in hybridization with the more general IAP probe (Lym-IAP) suggests that, as for normal lymphocytes, the major transcriptionally active IAP elements in the immunogenic sublines belong to the LS subfamily.

One cannot know how many genomic IAP elements are induced in the nontumorigenic (immunogenic) S49 sublines or how many of these are of the LS family. However, the findings that (i) sequence analysis of a few T-25-Adh and T-25 cDNA clones demonstrates LS motifs in the former but not in the latter, (ii) specific probes (for LS and nonapeptide sequences) demonstrate selective expression of 5.4-kb transcripts in T-25-Adh vs T-25 mRNAs, and (iii) a general probe (Lym-IAP, which supposedly recognizes all IAP transcripts) shows activation of 5.4-kb transcripts in T-25-Adh vs T-25 cells are all consistent with the notion that the LS family represents the majority of the IAP elements that undergo activation in T-25-Adh vs T-25 cells.

IAP elements may undergo transpositions and act as endogenous mutagens. Novel integration events of IAP are known to activate or inactivate growth factor genes, cytokine genes, proto-oncogenes, and tumor suppressor genes during tumor progression (Kuff, 1990). We found no difference in IAP integration sites between the immunogenic and the tumorigenic S49 sublines. The only subline that shows different integration sites is Rev-1T(a). This may be due to the UV irradiation applied during the process of their generation (Bergel *et al.,* 1993). As an example of UV irradiation causing retrotranspositions, it was shown (Servomaa and Rytomaa, 1990) that after UV treatment, 300,000 copies of L1 mobile DNA elements were inserted into apparently random locations in the cell genome. L1 elements frequently

occur in the 5′ flanks of IAP elements (K. Lueders, unpublished observation). It is therefore conceivable that UV treatment may cause insertion of L1 elements upstream of the LS1-IAP elements, thereby creating a new *Hin*dIII site. The Rev-1(Ta) pattern also has new shorter fragments that could have come from those that disappeared. The finding that no transpositions occurred in the other S49 sublines rules out the possibility that differences among integration sites in these cells play a role in regulating other gene activity or affect the expression of IAP itself.

Selective activation of IAP may stem from the preferential presence of, or the response to, transcription factors (not addressed in this study) or the methylation status of the LTR. Several studies have demonstrated that the transcriptional activation of IAP elements is influenced by the DNA methylation state (Dupressoir and Heidmann, 1996; Lueders *et al.,* 1993a; Lueders and Kuff, 1995; Mietz *et al.,* 1992; Walsh *et al.,* 1998). Methylation inhibits the binding of transcription factors to the IAP LTR and thereby inhibits its subsequent activation (Falzon and Kuff, 1991; Lamb *et al.,* 1991). Alterations in DNA methylation are common in cancer cells and are capable of directly modifying carcinogenesis (Jones, 1996; Warnecke and Bestor, 2000). In plasmacytoma cells, additional IAP elements are activated compared to normal LPS-stimulated lymphocytes (Lueders *et al.,* 1993a). In the present study, hypomethylation of individual IAP loci that was demonstrated in the immunogenic sublines is associated with enhanced IAP transcription in these cells. However, this is not the case for the tumorigenic S49 sublines, in which hypomethylation is not associated with enhanced IAP transcription.

Another point of interest arises from the sequence analysis of the IAP clones. In addition to the leucine zipper motif, the clones derived from T-25-Adh cells contain a sequence encoding a putative nonapeptide. Our attention has been drawn to this particular sequence, since this nonapeptide was previously shown to be immunogenic in LEC leukemia cells (de Bergeyck *et al.,* 1994). The nonapeptide sequence was recognized by CTLs when presented by H-2D^k molecules in LEC cells and it therefore has been considered a T-cell-defined tumor viral antigen (Van den Eynde and van der Bruggen, 1997). The correlation between the presence of the nonapeptide sequence and immunogenic phenotype is strengthened by the Northern blot findings that transcripts containing the nonapeptide are selectively expressed in the immunogenic S49 sublines. Indeed, antibodies to human IAP (HIAP) have been found in the serum of patients with autoimmune disorders, such as Graves' disease (Jaspan *et al.,* 1996), primary biliary cirrhosis (Mason *et al.,* 1998), lupus erythematosus (Mason *et al.,* 1998), idiopathic CD4 T lymphocytopenia (Garry *et al.,* 1996), and Sjogren's syndrome (Garry *et al.,* 1990). It is now believed that HIAP is etiologically linked to the above-mentioned autoimmune diseases.

Based on our findings and the studies cited above, it is tempting to speculate that the *in vitro* selection for cell adhesiveness causes up-regulation of specific IAP elements of the LS subfamily. Hypomethylation in the LTR region of these elements may result in enhanced transcription and expression of a specific nonapeptide. The *in vivo* expression of such a nonapeptide may enhance the immunizing potential of these cells against a subsequent challenge with suspension growing tumorigenic S49 cells. Is the expression of IAP instrumental in the regulation of cell adhesiveness, tumorigenicity, and immunogenicity in S49 cells or is it a marker tightly linked to the above phenotypes? Further experiments are needed to determine the primary targets of IAP element expression in the experimental model described in the present study. However, our results, whereby a full cycle from tumorigenic to nontumorigenic cells and back is associated with enhanced expression and lack thereof of specific IAP transcripts, suggest that, in S49 lymphoma, the former possibility may be the case. IAP may be seen, therefore, to act in an immunogenic and/or tumor suppressive capacity in these cells.

MATERIALS AND METHODS

Cell lines

Cells were grown at 37°C in Dulbecco's modified Eagle's medium containing 10% horse serum, penicillin (50 U/ml), and streptomycin (50 μg/ml), in a humidified atmosphere containing 5% CO_2.

T-25-Adh cells were derived from T-25 cells through spontaneous selection for cell substrate adherence as previously described (Hochman *et al.,* 1981, 1984). A similar selection procedure (from suspension to adhesive cells) was applied to X-18 cells (descendants of a different clone of S49 cells) subjected to 18 consecutive *in vivo* passages (Hochman *et al.,* 1981). Here again, the substrate adherent cell variants (named X-18-Adh) were nontumorigenic and immunogenic. T-25-Adh cells were subsequently subjected to UV mutagenesis and *in vitro* selection for suspension-borne revertants followed by *in vivo* selection for tumorigenic cells. These revertants, named Rev-1T(a), grew in suspension culture as single cells and were highly tumorigenic (similar to the parental T-25 cells) (Bergel *et al.,* 1993). In another independent UV selection experiment, we obtained suspension-borne revertants, named Rev-2-T-6 (Assaf *et al.,* 1997). Rev-2-T-6 cells grow in suspension culture, but as clumps, and develop progressive tumors, albeit with extended survival. Thus, while T-25, X-18, and Rev-1T(a) cells are highly tumorigenic (median survival of approximately 14, 23, and 17 days, respectively), Rev-2-T-6 cells develop progressive tumors that are less aggressive (median survival of approximately 80 days) and T-25-Adh and

X-18-Adh are nontumorigenic in syngeneic mice. All sublines were inoculated intraperitoneally into syngeneic BALB/c mice at a dose of 10^7 cells per mouse.

mRNA isolation

Total RNA was extracted with Trizol reagent (Gibco BRL, Life Technologies, Grand Island, NY) a mixture of guanidine isothiocyanate, phenol, and 2-mercaptoethanol, according to the manufacturer's instructions. mRNA was purified by the PolyAtract mRNA Isolation System (Promega, Madison, WI) that utilizes biotinylated oligo(dT), which hybridizes to streptavidin-coated magnetic beads.

mRNA differential display

mRNA was used for differential display, which was performed essentially as described by Welsh *et al.* (1992). mRNA (400 ng) was reverse-transcribed in a total reaction volume of 20 μl containing reverse transcriptase buffer [50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$], 10 mM DTT, a 200 μM concentration of each dNTP, 20 units of RNase inhibitor (RNasin Ribonuclease Inhibitor, Promega), a 1 μM concentration of the arbitrarily chosen 17-mer primer 5′-CCTCCGCGAGATCATCT-3′, and 200 units of reverse transcriptase (SuperScript II RNase H^-; Gibco BRL). The reaction was carried out at 42°C for 1 h followed by 95°C for 5 min. Half of the cDNA was then amplified by PCR in a total volume of 50 μl containing *Taq* polymerase buffer [10 mM Tris–HCl (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mg/ml BSA], a 1 μM concentration of the primer used for reverse transcription, 0.05 μCi/μl [α-^{32}P]dATP, and 1 unit of Red Hot polymerase (Advance Biotechnologies, Surrey, UK). The reaction mixture was subjected to the following cycling parameters: one cycle of 94°C for 5 min, 40°C for 5 min, and 72°C for 5 min followed by 30 high-stringency cycles: 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min followed by 72°C for 5 min. The amplified cDNAs were then separated on a 4% acrylamide denaturing gel. The reactions showing differentially expressed bands were repeated to ensure reproducibility.

Recovery of cDNA, reamplification, and TA cloning

The cDNA bands representing differentially expressed mRNAs were excised from the gel, rehydrated in 100 μl distilled H_2O for 10 min, and boiled for 10 min. Precipitation was performed in 250 μl ethanol containing 10% 3 M sodium acetate (pH 5.2) and 40 μg glycogen. The dried pellet was redissolved in 10 μl distilled H_2O. For each band, 3 μl cDNA was reamplified for 30 cycles in a total volume of 50 μl with the same primer and PCR conditions as in the initial PCR, except that no radioactive dATP was included. The reamplified product was cloned into the pCRII vector using the TA cloning system (InVitrogen, San Diego, CA).

Northern blot analysis

mRNA (1.5 μg) was electrophoresed in formaldehyde-containing 1% agarose gels and transferred to nylon membranes using standard techniques. The cDNA probe was labeled with [α-^{32}P]dCTP using a Random-Prime DNA labeling kit (Promega). Oligonucleotide probes were end-labeled with T4 polynucleotide kinase (Promega) and [γ-^{32}P]ATP. Oligonucleotide probes were as follows: LS1 probe, 5′-CTTACATCTTTTTGGGGCCAGAG-3′; LS2 probe, 5′-CTTACATCTTTATCGGGCCAGAG-3′; nonapeptide probe, 5′-GTATACTTACCGTTTCTCCTTGC-3′. For the Lym-IAP probe, the hybridization solution consisted of 50% formamide, 4× SSPE, 2× Denhardts' solution, 0.1% SDS, 12.5% dextran sulfate, and 100 μg/ml of salmon sperm DNA. Hybridization was performed at 42°C for 18 h, followed by one wash with 2× SSC, 0.5% SDS at 42°C and a second wash with 0.1× SSC, 0.1% SDS at 65°C. To determine the amounts of mRNA loaded on each lane, the blots were hybridized with a β-actin probe. Hybridization conditions for the oligonucleotide probes were done according to Lueders *et al.* (1993b).

DNA sequencing and analysis

Cloned reamplified PCR fragments were sequenced by Dye Terminator Cycle Sequencing with an ABI Prism 377 DNA Sequencer (Perkin–Elmer) and analyzed using Sequencing Analysis software. Identification of known sequences was performed using the Wisconsin Package version 9.0, Genetics Computer Group (GCG), Madison, Wisconsin.

RT-PCR

Primers were constructed using the sequence of a cloned fragment (see Lym-IAP, Fig. 4) isolated from the differential display reaction. The upstream primer was 5′-TCTCTCTTGCTTCTTACACTC-3′ (a primer that does not include LS sequence) from position 72–92 (Fig. 4). The downstream primer was 5′-TCCTTCTAATCTCTC-CCC-3′, complementary to the sequence in position 759–776 (Fig. 4) of the clone Lym-IAP. One microgram of total RNA was reverse-transcribed (as for the differential display), using a 1 μM concentration of each primer. The reaction was carried out at 37°C for 1 h and at 95°C for 5 min. Half of the cDNA was then amplified by PCR in a total volume of 50 μl containing *Taq* pol buffer (10 mM Tris–HCl (pH 9), 50 mM KCl, 0.1% Triton X-100), 1.5 mM $MgCl_2$, a 1 μM concentration of each of the primers used for reverse transcription, and 1 unit of *Taq* polymerase (Promega). The reaction mixture was subjected to the following cycling parameters for 30 cycles: 94°C for 1 min, 55°C for 0.5 min, and 72°C for 2 min, followed by 72°C for 5 min.

DNA isolation and hybridization of oligonucleotide probes to DNA in dried agarose gels

High-molecular-weight DNA isolation was previously described (Ehrlich *et al.,* 1994). Hybridization of IAP oligonucleotide probes to restriction enzyme-digested genomic DNAs in dried gels was described in detail (Lueders *et al.,* 1993b). DNAs were digested with *Hin*dIII, and one-half of each sample was then digested with *Hae*II. The LS1 and LS2 oligonucleotides were used for hybridization.

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REFERENCES

Assaf, N., Hasson, T., Hoch-Marchaim, H., Pe'er, J., Gnessin, H., Deckert-Schluter, M., Wiestler, O. D., and Hochman, J. (1997). An experimental model for infiltration of malignant lymphoma to the eye and brain. *Virchows Arch.* **431**(6), 459–467.

Bergel, M., Bhatia, K., Siwarski, D., Gutierrez, M., Hochman, J., and Huppi, K. (1993). Association of tumorigenic and nontumorigenic (immunogenic) variants in a mouse T-cell lymphoma with two distinct p53 mutations. *Mol. Carcinog.* **8**(4), 221–227.

Christy, R. J., Brown, A. R., Gourlie, B. B., and Huang, R. C. (1985). Nucleotide sequences of murine intracisternal A-particle gene LTRs have extensive variability within the R region. *Nucleic Acids Res.* **13**(1), 289–302.

de Bergeyck, V., De Plaen, E., Chomez, P., Boon, T., and Van Pel, A. (1994). An intracisternal A-particle sequence codes for an antigen recognized by syngeneic cytolytic T lymphocytes on a mouse spontaneous leukemia. *Eur. J. Immunol.* **24**(9), 2203–2212.

Dupressoir, A., and Heidmann, T. (1996). Germ line-specific expression of intracisternal A-particle retrotransposons in transgenic mice. *Mol. Cell. Biol.* **16**(8), 4495–4503.

Ehrlich, G., Ginzberg, D., Loewenstein, Y., Glick, D., Kerem, B., Ben-Ari, S., Zakut, H., and Soreq, H. (1994). Population diversity and distinct haplotype frequencies associated with ACHE and BCHE genes of Israeli Jews from trans-Caucasian Georgia and from Europe. *Genomics* **22,** 288–295.

Falzon, M., and Kuff, E. L. (1990). A variant binding sequence for transcription factor EBP-80 confers increased promoter activity on a retroviral long terminal repeat. *J. Biol. Chem.* **265**(22), 13084–13090.

Falzon, M., and Kuff, E. L. (1991). Binding of the transcription factor EBP-80 mediates the methylation response of an intracisternal A-particle long terminal repeat promoter. *Mol. Cell. Biol.* **11**(1), 117–125.

Garry, R. F., Fermin, C. D., Hart, D. J., Alexander, S. S., Donehower, L. A., and Luo-Zhang, H. (1990). Detection of a human intracisternal A-type retroviral particle antigenically related to HIV. *Science* **250**(4984), 1127–1129.

Garry, R. F., Fermin, C. D., Kohler, P. F., Markert, M. L., and Luo, H. (1996). Antibodies against retroviral proteins and nuclear antigens in a subset of idiopathic $CD4^+$ T lymphocytopenia patients. *AIDS Res. Hum. Retroviruses* **12**(10), 931–940.

Hochman, J., Katz, A., Levi, E., and Eshel, S. (1981). Substrate-adhering lymphoid cells show impaired tumorigenicity and increased immunogenicity. *Nature* **290,** 248–249.

Hochman, J., Levy, E., Mador, N., Gottesman, M. M., Shearer, G. M., and Okon, E. (1984). Cell adhesiveness is related to tumorigenicity in malignant lymphoid cells. *J. Cell Biol.* **99**(4, Pt. 1), 1282–1288.

Hochman, J., Park, S. S., Lazarovici, P., Bergel, M., and Gottesman, M. M. (1990). Monoclonal antibodies to immunogenic lymphoma cell variants displaying impaired neoplastic properties: Characterization and applications. *J. Natl. Cancer Inst.* **82**(23), 1821–1826.

Hoch-Marchaim, H., Hasson, T., Rorman, E., Cohen, S., and Hochman, J. (1998). Nucleolar localization of mouse mammary tumor virus proteins in T-cell lymphomas. *Virology* **242,** 246–254.

Jaspan, J. B., Sullivan, K., Garry, R. F., Lopez, M., Wolfe, M., Clejan, S., Yan, C., Tenenbaum, S., Sander, D. M., Ahmed, B., and Bryer-Ash, M. (1996). The interaction of a type A retroviral particle and class II human leukocyte antigen susceptibility genes in the pathogenesis of Graves' disease. *J. Clin. Endocrinol. Metab.* **81**(6), 2271–2279.

Jones, P. A. (1996). DNA methylation errors and cancer. *Cancer Res.* **56**(11), 2463–2467.

Krzyzosiak, W. J., Shindo-Okada, N., Teshima, H., Nakajima, K., and Nishimura, S. (1992). Isolation of genes specifically expressed in flat revertant cells derived from activated ras-transformed NIH 3T3 cells by treatment with azatyrosine. *Proc. Natl. Acad. Sci. USA* **89**(11), 4879–4883.

Kuff, E. L. (1990). Intracisternal A particles in mouse neoplasia. *Cancer Cells* **2**(12), 398–400.

Kuff, E. L., and Lueders, K. K. (1988). The intracisternal A-particle gene family: Structure and functional aspects. *Adv. Cancer Res.* **51,** 183–276.

Lamb, B. T., Satyamoorthy, K., Li, L., Solter, D., and Howe, C. C. (1991). CpG methylation of an endogenous retroviral enhancer inhibits transcription factor binding and activity. *Gene Expr.* **1**(3), 185–196.

Laskov, R., Sharir, H., Tzieger-Dickbuch, S., Hijazzi, M., Greenberg, A., and Ber, R. (1991). Suppression of the translocated myc gene and expression of intracisternal A-particle genes in tumorigenic and non-tumorigenic hybrids between murine myeloma and normal fibroblasts. *Int. J. Cancer* **48**(4), 574–582.

Lee, J. S., Haruna, T., Ishimoto, A., Honjo, T., and Yanagawa, S. (1999). Intracisternal type A particle-mediated activation of the Notch4/int3 gene in a mouse mammary tumor: Generation of truncated Notch4/int3 mRNAs by retroviral splicing events. *J. Virol.* **73**(6), 5166–5171.

Li, M., Muller, J., Rao, V., Hearing, V., Lueders, K., and Gorelik, E. (1996). Loss of intracisternal A-type retroviral particles in BL6 melanoma cells transfected with MHC class I genes. *J. Gen. Virol.* **77**(Pt 11), 2757–2765.

Lueders, K. K., Fewell, J. W., Morozov, V. E., and Kuff, E. L. (1993a). Selective expression of intracisternal A-particle genes in established mouse plasmacytomas. *Mol. Cell. Biol.* **13**(12), 7439–7446. [Published erratum appears in *Mol. Cell. Biol.* 1995 Jan;**15**(1), 590].

Lueders, K. K., Frankel, W. N., Mietz, J. A., and Kuff, E. L. (1993b). Genomic mapping of intracisternal A-particle proviral elements. *Mamm. Genome* **4**(2), 69–77.

Lueders, K. K., and Kuff, E. L. (1995). Intracisternal A-particle (IAP) genes show similar patterns of hypomethylation in established and primary mouse plasmacytomas. *Curr. Top. Microbiol. Immunol.* **194,** 405–414.

Mador, N., Falk, H., Bergel, M., Panet, A., and Hochman, J. (1997). Variant mouse lymphoma cells with modified response to interferon demonstrate enhanced immunogenicity. *Cancer Immunol. Immunother.* **44,** 249–257.

Mason, A. L., Xu, L., Guo, L., Munoz, S., Jaspan, J. B., Bryer-Ash, M., Cao, Y., Sander, D. M., Shoenfeld, Y., Ahmed, A., Van de Water, J., Gershwin, M. E., and Garry, R. F. (1998). Detection of retroviral antibodies in primary biliary cirrhosis and other idiopathic biliary disorders. *Lancet* **351**(9116), 1620–1624. [Published erratum appears in *Lancet* 1998 Jul 11;**352**(9122), 152] [see comments]

Mietz, J. A., Fewell, J. W., and Kuff, E. L. (1992). Selective activation of a discrete family of endogenous proviral elements in normal BALB/c lymphocytes. *Mol. Cell. Biol.* **12**(1), 220–228.

Mietz, J. A., Grossman, Z., Lueders, K. K., and Kuff, E. L. (1987). Nucleotide sequence of a complete mouse intracisternal A-particle genome: Relationship to known aspects of particle assembly and function. *J. Virol.* **61**(10), 3020–3029.

Mietz, J. A., and Kuff, E. L. (1992). Intracisternal A-particle-specific

oligonucleotides provide multilocus probes for genetic linkage studies in the mouse. *Mamm. Genome* **3**(8), 447–451.

Morgan, R. A., and Huang, R. C. (1984). Correlation of undermethylation of intracisternal A-particle genes with expression in murine plasmacytomas but not in NIH/3T3 embryo fibroblasts. *Cancer Res.* **44**(11), 5234–5241.

Ronai, Z., Robinson, R., Lee, G., Okin, E., Minarovits, J., Wirchubsky, Z., Klein, G., and Weinstein, I. B. (1992). Interaction of SEWA sarcoma cell proteins with the intracisternal A-type particle long terminal repeat DNA sequence. *Mol. Carcinog.* **5**(1), 81–89.

Servomaa, K., and Rytomaa, T. (1990). UV light and ionizing radiations cause programmed death of rat chloroleukaemia cells by inducing retropositions of a mobile DNA element (L1Rn). *Int. J. Radiat. Biol.* **57**(2), 331–343.

Van den Eynde, B. J., and van der Bruggen, P. (1997). T cell defined tumor antigens. *Curr. Opin. Immunol.* **9**(5), 684–693.

Walsh, C. P., Chaillet, J. R., and Bestor, T. H. (1998). Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. *Nat. Genet.* **20**(2), 116–117. [Letter]

Wang, X. Y., Steelman, L. S., and McCubrey, J. A. (1997). Abnormal activation of cytokine gene expression by intracisternal type A particle transposition: Effects of mutations that result in autocrine growth stimulation and malignant transformation. *Cytokines Cell Mol. Ther.* **3**(1), 3–19.

Warnecke, P. M., and Bestor, T. H. (2000). Cytosine methylation and human cancer. *Curr. Opin. Oncol.* **12**(1), 68–73.

Welsh, J., Chada, K., Dalal, S. S., Cheng, R., Ralph, D., and McClelland, M. (1992). Arbitrarily primed PCR fingerprinting of RNA. *Nucleic Acids Res.* **20,** 4965–4970.

Xiao, G. H., Jin, F., and Yeung, R. S. (1995). Germ-line Tsc2 mutation in a dominantly inherited cancer model defines a novel family of rat intracisternal-A particle elements. *Oncogene* **11**(1), 81–87.